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JOURNAL ARTICLE

Cytotoxicity to Alveolar Macrophages of Metal Oxides Adsorbed on Fly Ash

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ABSTRACT

Fly-ash particles fractionated into three size ranges (<2, 2 to 5, and 5 to 8 μm) and coated with various metal oxides were used to determine whether particle size and surface area are contributing factors to the in vitro toxicity of trace metals for alveolar macrophages (AM). When uncoated fly-ash particles and those coated with PbO, NiO, and MnO_2 in concentrations ranging from 50 to 1500 μg particles/ml were incubated with 10^6 AM/ml, reduced viability was observed on increase in concentration irrespective of type or size range of the particles. Within a given particle type and exposure concentration, decrease in particle size resulted in decreased viability and increased phagocytosis. The toxic effect was not due to the solubilization of the test elements into the incubating medium. The percentage of lead, nickel, or manganese adsorbed on the fly-ash particles varied within a relatively narrow range and was not affected by the particle size. Since the AM were exposed to the particles on a weight—dose per cell basis, their effective exposure load to the test elements was constant irrespective of the particle size. Thus the greater toxicity of the small particles appears to be due to surface interaction between particles and AM, and toxic effect is surface-area as well as dose related. These observations are further supported by decreases measured in total cellular protein levels and specific activity of lactate dehydrogenase.

The objective of this study was to examine the in vitro toxicity for alveolar macrophages (AM) of various sizes of particles coated with selected metal oxides. Fly-ash particles in three size ranges with oxides of lead, nickel, or manganese adsorbed onto their surface were

included in the studies. The parameters of toxicity were changes in viability, cell lysis, and activity of selected cellular enzymes.

METHODS

The fly-ash particles were fractionated by air classification into three size ranges, i.e., <2, 2 to 5, and 5 to 8 μm. The metals in the form of their hydroxides were then precipitated on the surface of the particles that were dispersed in an aqueous phase. After the particles had been filtrated and washed, they were heated to 675°C to convert the metal hydroxides to the respective oxides.

Alveolar macrophages were obtained from male albino New Zealand rabbits by tracheobronchial lavage.[1,2] The rabbits were killed by the injection of sodium pentobarbital into the marginal ear vein, and the lungs were lavaged in situ with warm (37°C) sterile saline through a catheter that was inserted into an incision in the trachea. The AM were centrifuged at 365 x *g* for 10 min and washed in Hanks' salt solution. Total cell counts were made in a hemocytometer, and viability was determined by the trypan-blue dye-exclusion technique.[3] Smears of air-dried cells were fixed in methanol and stained with Wright's stain for differential counts.

Medium 199 in Hanks' salt solution supplemented with *n*-2-hydroxyethylpiperazine-*n*'-2-ethanesulfonic acid (HEPES) buffer, gentamicin, and 10% heat-inactivated fetal-calf serum was used for incubation. Separate AM and particle suspensions were prepared in the medium at twice the projected exposure concentrations, and equal volumes of the two suspensions were mixed. The final AM concentration in the test suspensions was maintained constant at 10^6 AM/ml; the concentrations of the particles were varied to provide an exposure concentration ranging from 50 to 1500 μg particles 10^{-6} AM ml^{-1} medium.

The test suspensions were incubated in wells of disposable plastic cluster dishes that were placed on a rocker platform for agitation. After 21 hr of incubation at 37°C in a humidified 4% CO_2 atmosphere, the suspensions were transferred into siliconized test tubes. The AM attached to the cluster dishes were removed with rubber policemen and combined with the corresponding suspensions in the test tubes. Viability was determined by microscopic counting of 400 to 500 AM, and, from the same slides, the percentage of AM that had phagocytized was determined.

For determination of enzyme activity, the cells were first mixed with the particles in siliconized Erlenmeyer flasks, and then 10 ml of the test suspension containing 10^7 AM was distributed in four wells

of a disposable plastic cluster dish. This sample volume was used so that we would have sufficient numbers of AM required for the assays. After 21 hr of incubation, all cells were transferred into siliconized tubes, centrifuged at 365 x *g*, and washed in Hanks' salt solution three times. The cell pellet was resuspended in distilled water for osmotic shock, the volume adjusted to 2 ml, and the suspension placed in ice and sonicated with the microtip of an ultrasonic generator. The cell suspension was divided into two portions: one portion was used for enzyme assays after centrifugation at 63 x *g* to remove the metal particles, and the other portion was treated with sodium deoxycholate, the resulting lysate centrifuged at 10,000 x *g*, and the supernatant used for protein assay.

Enzyme activity was determined in triplicate with commercial assay kits. Acid phosphatase activity was determined by using the enzyme to hydrolyze *p*-nitrophenylphosphate at 37°C and measuring the liberated *p*-nitrophenol colorimetrically at 405 mμm. Beta-glucuronidase activity was measured by using phenolphthalein—glucuronic acid as a substrate and measuring the liberated phenolphthalein colorimetrically at 550 mμm. Lactate dehydrogenase (LDH) activity was analyzed by measuring the rate of oxidation of reduced nicotinamide adenine dinucleotide (NADH) at 366 mμm. This oxidation is proportional to the conversion by the enzyme of pyruvate to lactate. Total protein content was analyzed by the Lowry method[4] with the use of bovine serum albumin standard. Enzyme activities were calculated in milliunits per milligram of cellular protein. Changes in enzyme activity and in total cellular protein content were expressed as percentage of the corresponding controls. Since viability of the control AM was not affected by the 21-hr incubation, the viability values were reported directly as a percentage.

RESULTS

Viability of the macrophages was studied to establish the relative toxicity of the coated fly ash in all three particle size ranges. A close dose-response relationship between the concentration of the particles and viability was observed. Within each particle size viability of AM decreased when concentration of the particles was increased. This response appeared to be linear, providing a significant correlation coefficient ($r = 0.906$ to 0.975) on linear regression analysis of the data.

The viability values fitted by the least-square lines for the various size particles as well as the means of the actual percent viability are shown in Fig. 1. The data indicate that at a given concentration AM

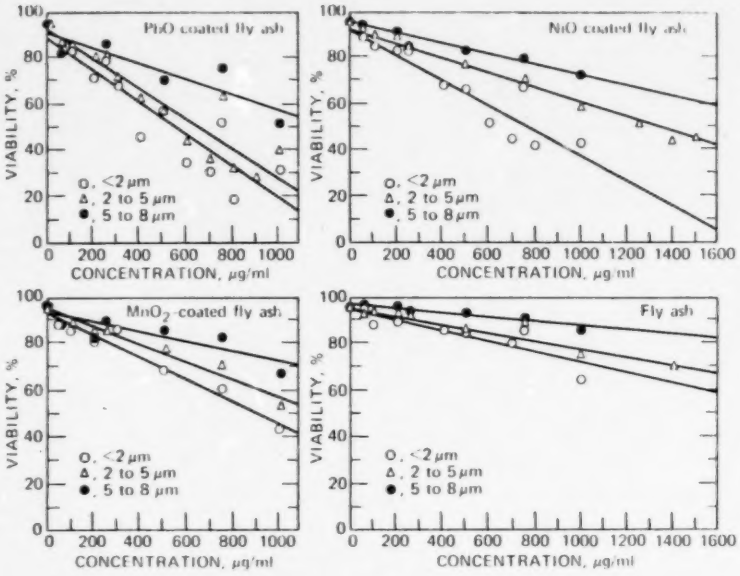

Fig. 1 Effect of concentration and size of fly-ash particles on viability of alveolar macrophages. Each point represents the mean of multiple cultures.

viability decreased with decrease in particle size of fly ash. Although uncoated fly-ash particles reduced AM viability to a much lesser extent, the size effect remained clearly evident.

Table 1 shows the estimated lethal concentrations (LC_{75}) of the various particles which reduce viability to 75% during a 21-hr exposure period. The results indicate that, irrespective of the particle size, PbO-coated fly ash was the most toxic, whereas the toxicity of NiO- and MnO_2-coated fly-ash particles was similar but lower than that of the PbO-coated fly ash. The uncoated fly-ash particles reduced viability at markedly higher concentrations than the coated particles.

The amounts of the test element deposited on the various fly-ash particles were analyzed by atomic absorption spectrophotometry. The data shown in Table 2 indicate that the percentage of adsorbed test metal varies within a relatively narrow range for all particles, and the variability within each particle type is even smaller. Therefore, on a weight-per-cell basis, the macrophages were exposed to fairly constant concentrations of the test metals irrespective of particle size. The fact that an increase in toxicity was observed with decreasing

TABLE 1

ESTIMATED CONCENTRATION OF PARTICLES REQUIRED TO REDUCE MACROPHAGE VIABILITY TO 75% DURING A 21-hr EXPOSURE

Fly ash	Particle concentration, $\mu g\ 10^{-6}$ AM ml^{-1}		
	Size range, <2 μm	Size range, 2 to 5 μm	Size range, 5 to 8 μm
Coated with			
PbO	180	270	470
NiO	320	550	870
MnO_2	375	510	860
Uncoated	870	1160	*

*Above the tested concentration range.

TABLE 2

PERCENT OF THE TEST ELEMENTS ADSORBED ON THE VARIOUS FLY-ASH CARRIER PARTICLES

Fly ash	Size range,* μm	Percent of test element
Coated with		
PbO	0 to 2	3.85
	2 to 5	3.84
	5 to 8	3.15
NiO	0 to 2	2.63
	2 to 5	2.91
	5 to 8	2.77
MnO_2	0 to 2	3.22
	2 to 5	3.67
	5 to 8	2.91
Uncoated	0 to 2	†
	2 to 5	†
	5 to 8	†

*Aerodynamic equivalent diameter.

†<0.03% lead and nickel and <0.01% manganese were in the uncoated fly ash.

particle size suggests that the toxic effect is due to concentration and to surface interaction between particles and alveolar macrophages. This is in agreement with the observations that within a given concentration the percentage of phagocytizing macrophages and the

apparent number of particles engulfed by the AM increase with decreasing particle size. Thus, since smaller particles can be phagocytized easier in larger numbers, they can provide more surface area for interaction with the lysosomal membrane or release of cytotoxic components within the phagolysosomes. Large particles do not affect AM viability as readily because they cannot be engulfed in similar numbers and because after engulfment less surface area is available to the particles to be acted on by the lysosomal hydrolases.

The highest exposure concentrations of the particles were incubated under conditions identical to those used in the dose-response experiments to assure that the cytotoxic effects were not due to solubilization of the test compounds in the culture medium. After incubation the particles were removed from the media by centrifugation and filtration through a 0.22-μm Millipore filter. After the particles had been incubated in the filtered medium for 21 hr, the viabilities of the AM did not differ from the controls, which indicates that the toxicity was not the result of solubilization of the test compounds in the extracellular medium.

Activity of acid phosphatase, LDH, and β-glucuronidase and the levels of total cellular protein in AM after 21 hr of incubation with the particles were studied to determine if a correlation with viability could be demonstrated or if a more sensitive indicator of cell dysfunction could be developed. The PbO- and NiO-coated <2-μm and 2- to 5-μm fly-ash particles were used at five concentrations for each particle type, which resulted in 30 to 80% viabilities. The results shown in Fig. 2 for <2-μm PbO-coated particles demonstrate that the decrease in viability was generally paralleled by the decreases in cellular protein levels (representing cell lysis), in specific activity of LDH, and, to a much lesser extent, in acid phosphatase. There appeared to be no change in the activity of β-glucuronidase. The decreases in viability, LDH activity, and protein levels showed significant linear dose-response relationships ($r = 0.990$, 0.980, and 0.933), and the estimated concentrations of the particles that reduced these experimental parameters to 50% were 450, 570, and 640 $\mu g\ 10^{-6}$ AM ml^{-1}, respectively.

Similar trends were found for the other particles examined. Although the doses of the compounds that resulted in 50% reduction of the enzymatic activities or cellular protein levels generally confirmed the toxic ranking determined during the viability experiments, the concentrations appeared to be higher than those affecting viability.

The PbO- and NiO-coated <2-μm and 2- to 5-μm fly-ash particles at concentrations that resulted in 50% reduction of viability were

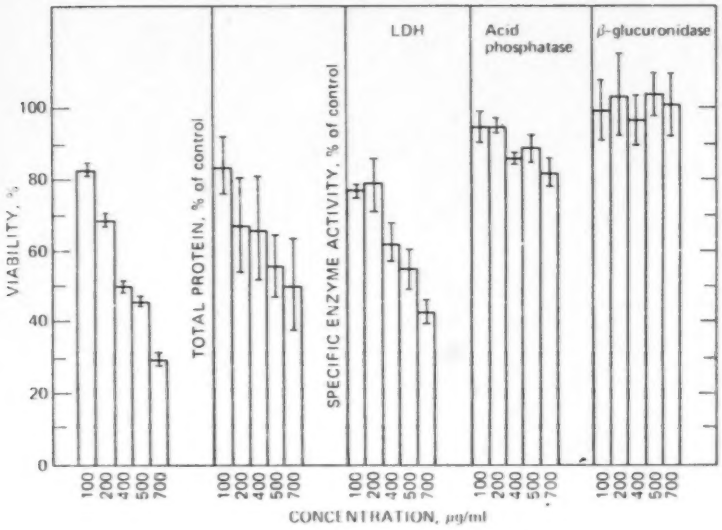

Fig. 2 Effect of exposure to PbO-coated fly-ash particles of $< 2\ \mu m$ on alveolar macrophage viability, total cellular protein content, and specific activity of LDH, acid phosphatase, and β-glucuronidase. Means ± standard error of three experiments are shown.

compared with uncoated fly-ash particles in the same size ranges and concentrations. Although the fly-ash particles per se affected all the experimental parameters used to evaluate toxicity, the decreases in these parameters were significantly smaller than they were after exposure to coated particles.

In summary, for a given test metal and a given particle concentration, toxic effects increased with decreasing particle size as determined by cell viability, total cellular protein levels, and specific activity of LDH. The toxic effect was not due to the solubilization of the metal compounds into the incubating medium. Since the percentage of lead, nickel, or manganese on the fly-ash particles was approximately the same for all particle sizes, the concentration of the test metals to which the AM were exposed was not affected by the particle size. Therefore the greater toxicity of smaller particles appeared to be due to the larger surface area. Thus the particle itself affects the toxicity of the compounds it transports into the intracellular milieu. Finally, the stability of the specific activity of the lysosomal hydrolases (acid phosphatase and β-glucuronidase) as compared to that of a soluble enzyme in the cytosol (LDH) suggests that the

release of hydrolytic enzymes in the cell may be a secondary event in the cytotoxic response. The determination of the sequence of events and mechanism of cell death will require further investigation.

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REFERENCES

1. O. N. Myrvik, E. S. Leake, and B. Fariss, Studies on Pulmonary Alveolar Macrophages from the Normal Rabbit: A Technique to Procure Them in a High State of Purity, *J. Immunol.*, 86: 128-132 (1961).
2. D. L. Coffin, D. E. Gardner, R. S. Holzman, and F. J. Wolock, Influence of Ozone on Pulmonary Cells, *Arch. Environ. Health*, 16: 633-636 (1968).
3. L. Weissbecker, R. D. Carpenter, P. C. Luchsinger, and T. S. Osdene, In Vitro Alveolar Macrophage Viability, *Arch. Environ. Health*, 18: 756-759 (1969).
4. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, Protein Measurement with Folin Phenol Reagent, *J. Biol. Chem.*, 193: 265-275 (1951).